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Effect of high dose vitamin D supplementation on antibody titres to heat shock protein

27 in adolescent girls

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Conflict of Interest

The authors have no conflict of interest to declare.

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Abstract

Objective: Although vitamin D deficiency is associated with several inflammatory conditions, there have been few studies on the effects of vitamin D supplementation on markers of oxidative stress and inflammation. The aim of the current study was to evaluate the effects of high dose vitamin D supplementation on heat shock protein 27 antibody (anti-Hsp27) titres in adolescent girls. **Methods:** Five hundred and fifty adolescent girls received vitamin D3 at a dose of 50,000 IU/week for 9 weeks. Demographic, clinical, biochemical markers including serum fasting blood glucose, lipid profile and anti-Hsp27 titers as well as hematological parameters including white blood cell count (WBC), and red blood cell distribution width (RDW) were determined in all the subjects at baseline and at the end of the study. **Results:** Serum vitamin D increased significantly, from 6.4 (4.2-9.6) ng/mL to 35.6 (25.8-47.5) ng/mL ($P < 0.001$) following the intervention. Furthermore, serum anti-Hsp27 titers were significantly lower after the 9 week vitamin D administration period [0.22(0.12-0.33) OD vs. 0.19(0.11-0.31) OD; $P=0.002$]. A significant correlation was found between serum anti-Hsp27 and RDW ($r=0.13$, $p=0.037$). The reduction in RDW values after intervention were particularly evident in subjects with the greatest increase in serum vitamin D levels. **Conclusions:** High-dose vitamin D supplementation was found to reduce antibody titers to Hsp 27. Further randomized placebo-controlled trials are warranted to determine the long time effect of vitamin D administration on the inflammatory process especially that associated with chronic disease.

Keywords: Vitamin D supplementation; inflammation; heat shock protein; RDW

1. Introduction

Several studies have shown that the inflammatory response, which may be associated with several chronic diseases, is characterized by the increase of pro-inflammatory cytokines, hematological inflammatory parameters and acute phase proteins such as C-reactive protein (CRP), fibrinogen and heat shock protein 27 (Hsp27) (Ghayour-Mobarhan, Saber, & Ferns, 2012; Marnell, Mold, & Du Clos, 2005; Medzhitov, 2008; Rodríguez-Hernández, Simental-Mendía, Rodríguez-Ramírez, & Reyes-Romero, 2013; Wang et al., 2011). Hsp27 is an intracellular molecular chaperone that facilitates the proper folding/refolding of newly synthesized/damaged proteins belong to the family of small Hsps (15–40 kDa) and constitutively expressed in cells in response to different stress such as oxidative stress (OS) (Ghayour-Mobarhan et al., 2012). Besides their role as molecular chaperones, Hsp70 and Hsp27 are overexpressed in response to injury-inducing signals and protect cells from apoptosis. Hsp27 also contributes to the modulation of inflammation (Martin, Mestril, Hilal-Dandan, Brunton, & Dillmann, 1997; Vander Heide, 2002; Yamboliev, Hedges, Mutnick, Adam, & Gerthoffer, 2000).

There is good data demonstrating that serum levels of antibody titres against Hsp27 (anti-Hsp27) are raised in patients with acute coronary syndrome, metabolic syndrome, coronary artery disease and gastrointestinal diseases (Dudeja, Vickers, & Saluja, 2009; Ghayour-Mobarhan et al., 2008; Pourghadamyari et al., 2011; Sahebkar et al., 2011). Irritable bowel syndrome (IBS) is a functional bowel disorder. There is accumulating evidence that there is an important role of stress and elevated stress responsiveness in the pathophysiology and clinical manifestation of IBS (Taché, Martinez, Million, & Rivier, 1999). There is an imbalance between pro- and anti-inflammatory cytokines as well as an increased population of mast cells in the ileum and colon is observed in IBS, which may be involved in the local intestinal inflammation (Törnblom, Lindberg, Nyberg, & Veress, 2002). Moreover, in

experimental models, the expression of Hsp27 and Hsp70 in the colon is confined to the surface epithelium which support the protective role of Hsps against injury in colon (Kojima et al., 2003). However, the association of serum anti Hsp27 antibody titers with IBS has not been reported previously.

Previous studies reported smoking is associated with higher Hsp27, Hsp70, Hsp72 and HspB8 expression (Nakayoshi et al., 2016; Ospelt et al., 2014; Volm, Mattern, & Stammeler, 1995) and also elevated Hsp72 IgG antibody levels (Prummel, Van Pareren, Barker, & Wiersinga, 1997) in humans. Although, the number of the enrolled cases was small in these studies.

Non-specific inflammatory markers include white blood cell (WBC) count and red cell distribution width (RDW), which are usually measured as components of the complete blood count (CBC) panel. Several studies have shown that an elevated WBC count is associated with several chronic diseases such as diabetes, cardiovascular disease and metabolic syndrome (Nakanishi, Suzuki, & Tatara, 2002; Nakanishi, Yoshida, Matsuo, Suzuki, & Tatara, 2002). RDW is also an indicator of heterogeneity of the erythrocytes, (Patel et al., 2009) has been demonstrated to strongly associated to higher risk of cardiovascular morbidity and mortality (Cavusoglu et al., 2010; Tonelli et al., 2008).

25-hydroxyvitamin D[25(OH)D] is known for its role in calcium/ phosphate homeostasis and the regulation of bone metabolism (Guillot, Semerano, Saidenberg-Kermanac'h, Falgarone, & Boissier, 2010). Other studies have found that low 25(OH) D concentrations are associated with several other conditions, including: metabolic syndrome, insulin resistance, hypertension (Reis, von Mühlen, Miller, Michos, & Appel, 2009), type 1 diabetes (Hyppönen, Läärä, Reunanen, Järvelin, & Virtanen, 2001), multiple sclerosis (Munger, Levin, Hollis, Howard, & Ascherio, 2006), rheumatoid arthritis (Merlino et al., 2004) and cancer (Lappe, Travers-Gustafson, Davies, Recker, & Heaney, 2007). Vitamin D

can impact on the inflammatory response by down-regulating the activation of nuclear factor (NF- κ B), thus decreasing the production of pro-inflammatory cytokines (D'ambrosio et al., 1998; Gysemans et al., 2005; Veldman, Cantorna, & DeLuca, 2000). The antioxidant properties of 25(OH)D operates through an increase of glutathione, and the inhibition of iron-associated lipid peroxidation of membranes, that has been shown in experimental and clinical studies (Bhardwaj, Bhattacharjee, Bhatnagar, & Tyagi, 2013; Garcion, Sindji, Leblondel, Brachet, & Darcy, 1999; Wiseman, 1993). There have been a limited number of studies that have reported an association between serum 25(OH) vitamin D and oxidative/inflammatory markers, but results have been inconsistent (Ngo, Sverdlov, McNeil, & Horowitz, 2010; Timar et al., 2019; Yildirim, Hur, & Kokturk, 2013).

To best our knowledge, there have been no previous reports on the effect of vitamin D supplementation on oxidative/inflammatory marker, anti-Hsp27 titres. Thus, the goal of this study was two-fold: (i) to investigate whether autoantibodies levels against the stress-inducible Hsp27 antigen related to presence of IBS or exposure to passive smoking; (ii) to assess the effect of high dose vitamin D supplements on anti-Hsp27 and their association with other haematological inflammatory in a large number of adolescent girls.

2. Methods

2.1. Study design and population

The current intervention was undertaken on adolescent girls with vitamin D deficiency ($<20\text{ng/mL}$), aged between 12 and 17 years as described previously (Bahrami et al., 2018; Bahrami et al., 2017). Overall, 550 girls completed the intervention after a dropout rate of 4.8% (Figure 1). Participants were selected using a cluster randomized sampling method from various regions within the city of Mashhad, in north-eastern Iran. In the screening phase, subjects were screened for the inclusion criteria that were as follows: absence of any autoimmune diseases; metabolic bone disorders; cardiovascular diseases; thyroid,

parathyroid, or adrenal diseases; hepatic failure; malabsorption; renal diseases; or cancer. Adolescents taking anti-inflammatory, antidepressant, antidiabetic, or antiobesity drugs; vitamin D or calcium supplement; or hormone therapy during the past 6 months were excluded. The study protocol was approved by the Ethical Committee of Mashhad University of Medical Sciences, Iran (IRCT201509047117N7). Informed consent was obtained from all participants.

Eligible girls received a 50,000 IU soft gel capsule vitamin D once a week for 9 weeks, in accordance with the guidelines by Holick et al (Holick et al., 2011). We requested that all subjects did not take any additional dietary supplements, and continued to consume their usual food intake. The consumption of the vitamin D supplementation was assessed each week by a face-to-face interview or phone call.

2.2. Demographic, anthropometric, and metabolic data

For all subjects, weight (in kg), height (in cm) were measured and body mass index (in kg/m²) were calculated. Blood pressure was recorded using a digital sphygmomanometer (Omron M3, Kyoto, Japan) with the subjects in a supine position after a 5-min rest. The pulse rate was assessed twice with 5-min intervals by an expert nurse and then, the average of two measurements was applied. Questions related to lifestyle/ environmental factors were also asked, e.g. “How many hours per day are you exposed to cigarettes/tobacco smoke?”

2.3. Haematological and biochemical measurements

Blood samples for each subject were collected at baseline and at the end of treatment period after a 12-h fasting. We determined serum fasting blood glucose (FBG) and a full fasted lipid profile, consisting of: serum total cholesterol (TC), triglyceride(TG), high density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C) using commercial kits (Pars Azmun, Tehran, Iran) by the BT-3000 auto-analyser (Biotechnica, Rome, Italy). Serum hs-CRP concentration was evaluated by immunoturbidimetry method, with a detection limit

of 0.06 mg/L (Pars Azmun, Karaj, Iran) (Kazemi-Bajestani et al., 2017). Serum 25 (OH) D values was quantified using an Electrochemi-luminescence method (ECL, Roche, Basel, Switzerland). WBC and RDW were assessed as part of the automated CBC by using an auto hematology analyzer (Sysmex K-800).

2.3.1. Serum anti-Hsp27 assay

An in-house ELISA assay was applied to determine serum anti-Hsp27 levels as described previously (Ghayour-Mobarhan et al., 2008). In brief, microtitre plates were coated with recombinant human Hsp27. The wells were washed thrice with phosphate buffered saline (PBS), serum was diluted 1:100 with 2% goat serum in PBS (for blocking and reduction of nonspecific binding), added to each well in duplicate and incubated for 30 min at room temperature (RT). After washing 100 μ L of peroxidase conjugated-goat anti-human IgG (Sigma-Aldrich, Poole, UK) diluted with 2% goat serum in PBS. Subsequently, 100 μ L of tetramethylbenzidine (TMB) substrate was added per well and plates incubated for 15 minutes in the dark at room temperature. The reaction was terminated by adding 50 μ L of 2M HCl to each well and optical density was read at 450 nm with a reference wavelength of 620 nm.

2.4. Evaluation of IBS

To evaluation of the presence of IBS, we used a validated Persian version of the Rome III questionnaire (Sorouri et al., 2010). IBS is identified by abdominal pain or discomfort (at least 3 days per month within last 12 weeks) together with two or more of the following criteria: 1) pain improvement with defecation at least sometimes 2) changes in number of evacuations and 3) distortion in the stool for (appearance).

2.5. Statistical analysis

The normality of data was assessed using the Kolmogorov-Smirnov test. Descriptive statistics including mean \pm standard deviation (SD) or median (interquartile range IQR) was determined for normally or non-normally distributed variables, respectively. One-way

analysis of variance (one-way ANOVA) or Kruskal-Wallis test or chi-square/Fischer's exact test was also used to compare the demographic, anthropometric and biochemical parameters of the studied population in the different tertiles of anti-Hsp27. Multinomial logistic regression was recruited to evaluate the association between anti-Hsp27 and the other variables (adjusted for age and BMI). Participants in the 1st tertile (lowest level of anti-Hsp27) were defined as the reference group. Statistical significance for the variables compared in the participants pre- and post-vitamin D supplementation was analyzed using paired sample t-test (normally distributed variables) or Wilcoxon test (non-normally distributed variables). A p-value<0.05 was considered statistically significant. All statistical analyses were performed using Statistical 100 Package for Social Sciences version 16 (SPSS Inc., Chicago, Illinois, USA).

3. Results

3.1. Baseline characteristics of study population and relation to anti-Hsp27

Of the 889 girls invited to participate in the present study, 577 were eligible for inclusion. All 577 subjects received capsules of 50,000 IU of vitamin D, and 550 adolescents girls with the average age was 14.56 ± 1.53 years were finally completed the study (Figure 1).

General and clinical characteristics of the studied population across tertile categories of anti-Hsp27 levels are presented in Table 1. The median (interquartile ranges) of anti-Hsp27 were 0.10(0.01-0.155) OD in the first tertile, 0.21(0.155-0.285) OD in the second tertile, and 0.38(0.285-0.95) OD in the third tertiles, respectively (Table 1). The mean value of several cardiovascular risk factors include age, systolic and diastolic blood pressure, FBG, HDL-C, LDL-C, TC, TG were not differed between tertiles ($P > 0.05$). The percentage of those exposed to the smoking increased significantly from the 1st quartile (0%) to the 3rd tertile (41.7%) of the anti-Hsp27 tertiles ($p < 0.001$). The mean levels for RDW in the third tertile were significantly higher than other tertiles ($13.2 \pm 1.8\%$ in 3rd vs. $12.9 \pm 1.2\%$ in 2nd, and

12.7±0.87 % in 1st ; P=0.029; Table 1). Moreover, the frequency of IBS was significantly higher among 2nd and 3rd tertile of serum anti-hsp27 categories compared to the 1st tertile (22.9% vs. 19.5% vs. and 10.2% respectively). A significant Pearson's correlation was found between anti-Hsp27 and RDW (Figure 2).

The first tertile of serum anti-Hsp27 categories was set to be the reference group in the multivariate analysis. In multinomial logistic regression (after adjustment), there were significant association between passive smoking and first anti-Hsp27 tertile with 2nd and 3rd anti-Hsp27 tertile, respectively (OR =1.09, P<0.01/OR= 16.7, P< 0.01; Table 2).

3.2. Effect of vitamin D supplementation on serum anti-Hsp 27

Serum vitamin D significantly increased from 6.4(4.2-9.6) ng/mL to 35.6(25.8-47.5) ng/mL (P<0.001) after the 9 week intervention period. Furthermore, serum anti-Hsp27 was significantly lower after the 9 week vitamin D supplementation [0.22(0.12-0.33) OD vs. 0.19(0.11-0.31) OD; P=0.002].

The differences in BMI and inflammatory markers in different tertiles of magnitude of response to vitamin D are presented in Table 3. The median (interquartile ranges) of magnitude to response to vitamin D supplementation were 11.1(−19.5-20.9) ng/mL in the first tertile, 27.7(20.9-35.1) ng/mL and 44(35.1-66.4) ng/mL in the second and third tertiles, respectively (Table 3). RDW was significantly decreased in 2nd, and 3rd tertiles of response to treatment with vitamin D (P=0.001 and P=0.004, respectively) post-trial. The anti-Hsp27 was only significantly reduced in the 2nd tertile of response to treatment with vitamin D (P=0.001).

4. Discussion

To the best of our knowledge this is the first study to examine the impact of vitamin D supplementation on anti-Hsp27 in a large population of adolescent girls. Our results suggest that a high dose supplementation of vitamin D can lead to reduction in markers of

inflammation, as assessed by anti-Hsp27 and haematological inflammatory biomarker, RDW. It is estimated that nearly half of the Iranian population has suboptimal 25(OH)D levels (Hovsepian, Amini, Aminorroaya, Amini, & Iraj, 2011). Vitamin D deficiency might affect mortality by different mechanisms, also individuals with vitamin D deficiency tend to have impaired immunity and have higher levels of systemic inflammatory markers (Quraishi et al., 2013). Several studies have demonstrated the effect of vitamin D supplementation on inflammatory process (Beilfuss, Berg, Sneve, Jorde, & Kamycheva, 2012; Tabatabaeizadeh et al., 2017). Tabatabaeizade *et al.* observed that mega dose supplementation of vitamin D reduced Neutrophil-to-lymphocyte ratio (NLR) distribution and high-sensitivity (hs-CRP) level in adolescent girls (Tabatabaeizadeh et al., 2017). Year-Long et al reported a significant fall in serum IL-6 after 1 year vitamin D supplementation in overweight and obese subjects (Beilfuss et al., 2012). This effect can be explained by several mechanisms including: decreasing lymphocyte proliferation, reduction of pro-inflammatory cytokine and inhibition of NF- κ B production (Hoeck & Pall, 2011). It has been reported that supplementation of vitamin D can raise the level of active form of vitamin D (1,25-dihydroxyvitamin D₃) that can inhibit NF- κ B in murine macrophage cells and smooth muscle cells in human airways (Song, Hong, Liu, Lin, & Lai, 2013).

There have been few studies investigating the anti-inflammatory effects of nutrients. Ebrahimi et al. evaluated the effect of omega-3 supplementation on serum levels of anti-Hsp27 in subjects with metabolic syndrome. The authors observed that low doses of omega-3 supplementation can significantly reduce serum anti-Hsp27 (Ebrahimi et al., 2009). Moreover, in a study on major thalassemia subjects, zinc supplementation can have effects on the reduction of serum anti-Hsp27 titers (Ghahramanlu et al., 2014). But curcuminoid supplementation had no significant effect on serum concentrations of anti-Hsp27 in obese individuals (Sahebkar et al., 2013). Hsps are protective against wide spectra of stressors

which causes proteins denaturation and possibly reduce cell survival. Thermal or oxidative stress, environmental stress, inflammation, infections and microbial toxins, hypoxia, irradiation, chemokine/cytokines, ischemia and psychopathological stress can cause an induction in Hsp production (Hayase et al., 2003; Wu & Tanguay, 2006). Moreover, Hsps have immune activities in which Hsp27 is usually up-regulated in cells undergo OS to defense from damaging stressors. OS through production of reactive oxygen species (ROS) deteriorate the cellular integrity via oxidation of membranous lipids/proteins and DNA resulting to the cell faint (Tsan & Gao, 2009).

Otero et al. observed that low vitamin D status was correlated with the increased probability of elevated RDW. Furthermore when comparing subjects to 25(OH)D levels <30 ng/mL and levels ≥ 30 ng/mL, their results showed a noticeable significance (Otero, Monlezun, Christopher, Camargo, & Quraishi). This can be explained at least in part by vitamin D modifying the inflammatory responses by inhibiting the production of tumour necrosis factor (TNF)- α , interleukin (IL)-2 and production of interferon as well as by upregulating anti-inflammatory cytokines, such as IL-10 (Quraishi, Bittner, Blum, Hutter, & Camargo, 2014). Low vitamin D status was reported to be associated with increase inflammatory markers and suppress red blood cell (RBC) maturation. Additionally, pro-inflammatory cytokines decreases half-life of RBC and deforms their membranes (Fujita et al., 2015). In turn a raise in size heterogeneity of RBCs and therefore, RDW is shown (Fujita et al., 2015). In our study, we found that there was positive correlation between RDW and anti-Hsp27. As we know, both RDW and anti-Hsp27 associated with inflammation and in this situation there is an inflammatory process (Fujita et al., 2015; Ghayour-Mobarhan et al., 2008). Inflammation might result to elevation of RDW levels not only via deregulation of iron metabolism but also through inhibiting erythropoietin gene expression, preventing proliferation of erythroid progenitor cells, reducing erythropoietin receptor amplification, attenuating erythrocyte

circulatory half-life. In this way inflammation may contributed in anisocytosis from release and spreading of immature RBCs to the peripheral(Öztürk et al., 2013).

The present study is the first linking anti-Hsp27 to passive smoking and IBS. Previous research has indicated that serum anti-Hsp antibody levels may be evidence for an inflammatory status that predisposes to cardiovascular disease and IBS (Ghayour-Mobarhan et al., 2012; Zhou et al., 2015). Also other inflammatory situation such as cigarettes smoking and passive smoking induces Hsps (Newkirk et al., 2012). IBS is one of the most common chronic psycho/gastrointestinal disorders which affects approximately 15–20% of adolescents. Both serological cytokine studies and histological specimens have demonstrated low grade mucosal inflammation and chronic alteration of the immune response in IBS cases and immune activation possibly contributed in the pathophysiology of IBS (Öhman & Simrén, 2010; Spiller et al., 2000). In agreement with our result, He *et al.* reported significantly higher serum Hsp70 in individuals with IBS (He et al., 2018). Studies in mice demonstrated that serum Hsp70 concentrations are associated with post-infection IBS (Zhou et al., 2015). In normal intestinal mucosa, Hsp27 are constitutively expressed in the colonic surface epithelial cells, regions that are constantly encountered to inducing stimuli from enteric bacterial flora which involves in colonic epithelial resistance to toxins of pathogenic microorganisms and inflammation-related stress. Under inflammatory conditions associated with IBS, the down-expression of crucial cytoprotective proteins, an induction of Hsps, via translational suppression could have adverse outcome (Hu et al., 2007). Hsps can inhibit the generation of proinflammatory cytokine or stimulate production of anti-inflammatory cytokines and thus regulate the intensity of the immune response(Barbatis & Tsopanomichalou, 2009).

The exact mechanisms of the association between anti-Hsp27 levels and passive smoking not still obvious; OS may play an predominant role in connection of Hsp27 and cigarette smoking (Aksoy et al., 2012; Forchhammer et al., 2012).

The strength of our study is the population-based study design; additionally, all laboratory measurements were done by certified examiners following standardised protocols. The major limitation of the present study was the absence of a placebo group to be compared to the intervention group because of ethical considerations. So, it is need to interpret our data with caution.

5. Conclusion

The findings of the present study indicated that vitamin D3 supplementation (50000 IU/week for 90 days) leads to a significant reduction in serum anti-Hsp27 concentrations which highlighted the anti-inflammatory/anti-oxidative effect of vitamin D. While these findings need to be confirmed in future by longer durations of follow-up and larger scale trials.

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Table 1. Demographic and biochemical characteristics of individuals by tertile of serum anti-heat shock protein 27

	1st Tertile (n=184)	2nd Tertile(n=183)	3rd Tertile (n=183)	P*
	0.10(0.01-0.155)OD	0.21(0.155-0.285)OD	0.38(0.285-0.95)OD	
Age (year)	14.8±1.4	14.6±1.5	14.6±1.5	0.33
BMI (kg/m ²)	20.3±2.3	19.8±2.4	20.1±2.1	0.08
Physical activity (MET.h/week)	45.7±3.8	45.4±3.9	45.0±2.8	0.26
SBP(mmHg)	96.6±13.5	97.8±13.6	96.3±14.6	0.63
DBP(mmHg)	64.4±12.9	63.4±12.6	61.4±14.0	0.13
Passive smoker (%) (yes)	0(0)	0(0)	76(41.7)	<0.001
IBS (%) (yes)	19(10.2)	42(22.9)	36(19.5)	0.015
FBG (mg/dl)	87.4±11.9	86.3±12.8	84.4±10.5	0.093
HDL-C (mg/dl)	47.9±8.8	47.4±8.3	47.7±8.6	0.88
LDL-C (mg/dl)	98.4±26.2	94.5±22.4	99.8±26.0	0.204
TC(mg/dl)	159.9±31.5	156.7±26.0	161.4±30.1	0.415
TG(mg/dl)	75.0(54.9-93.0)	70.0(55.0-97.5)	71.0(56.0-94.0)	0.89
WBC(10 ⁹ /L)	6.0±1.9	6.4±1.7	6.4±2.2	0.24
RDW (%)	12.7±0.87	12.9±1.2	13.2±1.8	0.029
Hs-CRP (mg/l)	1.3(0.4-1.6)	2.2(0.47-1.6)	1.7(0.4-1.9)	0.67

Data are given as mean±SD for normally distribution variables or median (Interquartile range) for non-normally distributed variables or number (%) for categorical variables.

Obtained from one-way ANOVA/ Kruskal-Wallis test or chi-square/Fischer's exact test.

Abbreviations: BMI: body mass index; SBP: systolic blood pressure; DBP: diastolic blood pressure; IBS: irritable bowel syndrome; FBG: fasting blood glucose; HDL-C: high-density lipoprotein cholesterol; LDL-C: low-density lipoprotein cholesterol; TC: total cholesterol; TG: triglyceride; WBC: white blood cell; RDW: red blood cell distribution width; HSP: heat shock protein; hs-CRP: high sensitivity C-reactive protein.

Table 2. Multinomial logistic regression: Odds of variables according to the serum anti-heat shock protein 27 Tertiles

Variables	Reference group and 2nd Tertile	Reference group and 3rd Tertile
Passive smoker (%) (yes)	1.1(1.05-1.15) *	16.7(15.8-18.7) *
IBS (%) (yes)	2.5(1.1-5.8) *	3.4(1.4-8.2) **
RDW (%)	1.2(0.89-1.58)	1.2(0.93-1.7)
-Adjusted for age and BMI. *p < 0.05; **p < 0.01. IBS: Irritable Bowel Syndrome; RDW: red blood cell distribution width.		

Table 3. Comparison of inflammatory markers before and after supplementation with vitamin D among different tertiles of response to treatment.

		Response to treatment			P^α
		1st Tertile (n=182) 11.1 (-19.5-20.9) ng/mL	2nd Tertile (n=184) 27.7(20.9-35.1) ng/mL	3rd Tertile (n=184) 44(35.1-66.4) ng/mL	
WBC (10⁹/L)	Pre-intervention	6.1±1.7	6.2±1.9	5.9±1.5	0.60
	Post-intervention	5.7±1.3	6.3±1.3	5.8±1.6	0.028
P^β		0.32	0.52	0.58	
RDW (%)	Pre-intervention	12.9±2.1	13.1±1.5	12.8±0.9	0.23
	Post-intervention	12.6±0.7	12.7±1.0	12.6±0.77	0.72
P^β		0.33	0.001	0.004	
BMI	Pre-intervention	20.5±2.5	20.3±2.2	19.6±2.4	0.015
	Post-intervention	20.5±2.2	20.3±2.3	19.5±2.4	0.005
P^β		0.92	0.45	0.92	
hs-CRP (mg/l)	Pre-intervention	0.87(0.38-1.28)	1.0(0.45-2.15)	0.93(0.48-2.1)	0.19
	Post-intervention	0.76(0.27-1.83)	1.3(0.55-1.99)	0.91(0.50-1.6)	0.45
P^γ		0.50	0.57	0.67	
Anti-HSP27 (OD)	Pre-intervention	0.22(0.13-0.32)	0.25(0.13-0.34)	0.21(0.12-0.34)	0.57
	Post-intervention	0.21(0.11-0.33)	0.17(0.11-0.29)	0.20(0.13-0.34)	0.46
P^γ		0.78	0.001	0.23	

Abbreviations: WBC: white blood cell; RDW: red blood cell distribution width; HSP: heat shock protein; hs-CRP: high sensitivity C-reactive protein.

^α By using ANOVA test or Kruskal-Wallis test.

^β By using paired sample t-test.

^γ By using Wilcoxon test.

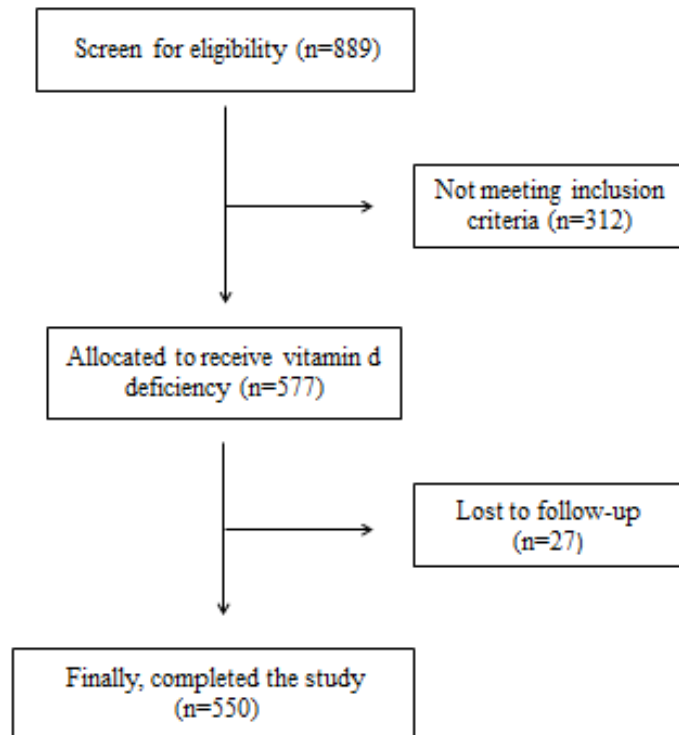


Figure 1. Flow diagram of trial.

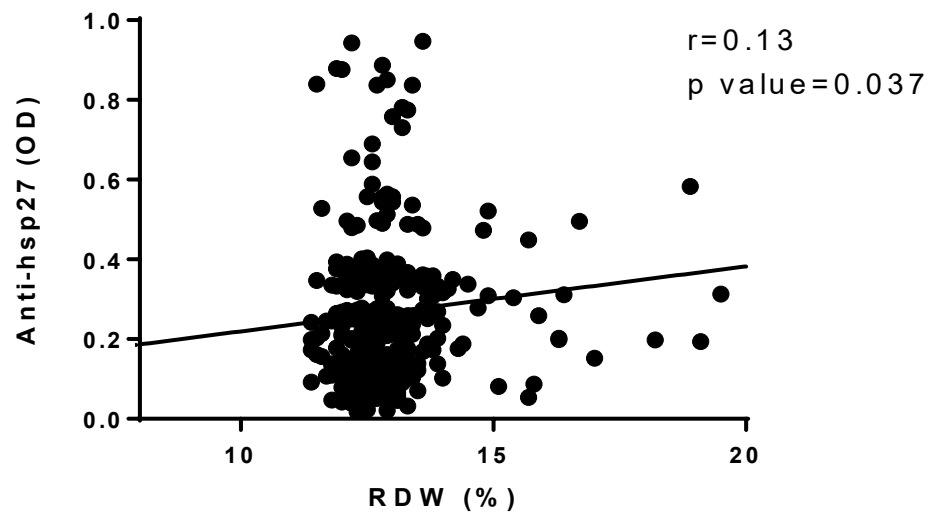


Figure 2. Correlation coefficient between red blood cell distribution width and anti-heat shock protein 27.